岩手県立大学看護学部紀要 9 ：13－20，2007
Journal of the Faculty of Nursing, Iwate Prefectural University

Prediction of efficacy of lamivudine therapy for chronic type B liver diseases：studies based on changes in HBV-DNA amount and YMDD motif

Kazuyoshi Ishikawa*1, Chihoko Nakakarumai*2,
Chie Matsumoto*1, Sayaka Ooe*1, Naoko Isobe*3

Abstract

Lamivudine was orally administered at 100 mg/day and was observed for more than 1 year in 5 patients, comprising 4 patients with chronic type B hepatitis and 1 patient with decompensated liver cirrhosis. The relationship between clinical course and frequency of mutant virus emergence and the mutation pattern was investigated in these patients. All 5 patients were male, with a mean age of 55.0 ± 6.9 years and a mean observation period of 39.0 ± 15.5 months. Before the start of lamivudine administration, mean HBV-DNA level was ranging from 5.8 to 6.9 LGE/mL and mean ALT level, from 33 to 471 IU/L. Only the patient with liver cirrhosis was positive for HBeAg. In all cases, genotype was C. In 4 of the 5 patients, YMDD motif before the start of lamivudine administration was wild-type, but mutant type YVDD was present in 1 patient who had been treated with low-dose intermittent administration of lamivudine prior to changing to the 100 mg/day treatment. In 2 of the 4 patients in whom YMDD motif was initially wild type, YVDD variant strains emerged at 45 months and 8 months, respectively, after the start of lamivudine administration. Within 1 month after lamivudine treatment was started, HBV-DNA level in each patient fell below the limit of detection, and within 3 months ALT levels had also normalized. In 2 of 3 patients in whom YVDD mutant strains emerged, HBV-DNA level remained below the limit of detection, ALT was normalized and clinical condition remained stable despite emergence of a variant strain. During the course of observation for the third of these patients, ALT aggravated twice together with an upward rebound in HBV-DNA level. In both patients who continuously showed wild-type YMDD motif, HBV-DNA level remained below the limit of detection and ALT also continued to be normal. Analysis of the course for patients in whom YVDD variants emerged revealed cases with a time-phase discrepancy between fluctuations in variants in domain B and fluctuations in YMDD variants. This suggests the possibility that this discrepancy influences the therapeutic effects of lamivudine.

Keywords：lamivudine, YMDD motif, HBV-DNA, breakthrough hepatitis

1. Introduction

Lamivudine ((−)2'-deoxy-3'-thiacytidine), a new therapeutic drug for chronic type B liver disease, is one of the nucleoside analogues which acts on as a antiviral agent. Lamivudine is phosphorylated inside cells, and then targets the polymerase/reverse transcriptase region of the P gene of HBV-DNA. The drug then stops proliferation of the virus through competitive inhibition and arrest of DNA elongation. As a result, lamivudine has attracted attention as a drug that expresses clinical efficacy[1,2]. However, it is of great concern that in long-term use of the drug, resistant viral strains can emerge, drug efficacy weakens and breakthrough hepatitis occurs. This state can lead to a clinical dilemma as the physician agonizes over whether lamivudine administration should be continued or a switch should be made to another drug regimen[3,4].

We report herein the results of our investiga-

＊1 Faculty of Nursing, Iwate Prefectural University
＊2 Iwate Seiwa Hospital
＊3 Iwate Health Service Association

tions into the relationship between clinical course and frequency of mutant virus emergence and mutation pattern in patients with chronic type B liver disease who had received long-term administration of lamivudine.

2. Materials and methods

2. 1. Subjects

Lamivudine was orally administered at 100 mg/day in 5 patients, comprising 4 chronic hepatitis and 1 decompensated cirrhosis who had been observed for more than 1 year. All 5 patients were male, with a mean age of 55.0 ± 6.9 years (range, 47－65 years) and a mean observation period of 39.0 ± 15.5 months (range, 13－52 months). One patient had previously been administered lamivudine 150 mg twice a week, which was then increased to 3 times/week, for a total of 32 months of treatment, before switching to the 100 mg/day regimen.

2. 2. Quantification of HBV-DNA

HBV DNA was quantified using a TMA kit (Chugai Diagnostic Science, Tokyo, Japan) according to the methods of Kamisango et al.[5] Briefly, from a 300-μ Lserum sample, the HBV-specific base sequence was amplified as RNA mainly using 2 enzymes, 2 primers and a substrate. Furthermore, RNA was quantified based on chemiluminescence induced by single-stranded DNA probes labeled with acridinium ester that were complementary to the amplified RNA. Results are expressed as log genome equivalents (LGE) per milliliter.

2. 3. Determination of serum HBeAg and anti-HBe

Serum HBeAg and anti-HBe were determined by enzyme-linked immunosorbent assay.

2. 4. HBV genotyping

HBV genotyping were performed using restriction fragment length polymorphism (RFLP) as described by Mizokami et al.[6] In brief, from a 100 μl of serum sample, DNA was extracted using SMITEST EX-R & D (Genome Science Laboratories). Next, 85 S gene sequences were aligned and analyzed to determine genotype-specific conserved sequences. After identifying restriction enzyme sites, HBV DNA extrac t s were amplified by nested PCR with the first-round (sense: HBMF 1; antisense: HBMR 2 primers) and then the second-round (inner sense: HBMF 2 and antisense: HBMR 2 primers). Restriction digestions were performed using second-round PCR product and reactions were undertaken with 10 units of AlwI, HphI, NciI, NlaIV or EarI (New England BioLabs.). Digested PCR products were electrophoresed on agarose gel containing ethidium bromide. The RFLP pattern was then evaluated under ultraviolet light.

2. 5. Determination in YMDD motif and sequences concerned in domain B

DNA amplification and direct sequencing in the YMDD motif and sequences concerned in domain B (nt 736－747, nt 664－672) were performed at least once in three months during follow-up. Briefly, an ALFexpress automated sequencer (Amersham Bioscience) was used for direct sequencing of PCR products. Primer sequences used comprised a 1st primer (amplification size, 484 bp): 5'-TCC1TGCTGCTATG CCTCAC-3' (nt 411－430) and 5'-TTCCGTCGA CATATCCCATGAAGTTAAGGGA-3' (nt 895－865); and a 2nd primer (amplification size, 289 bp): 5'-TCGGACGGAAACTGCACTTG-3' (nt 581－660), 5'-AAGGGAGTAGCCCCAACGTT-3' (nt 870－851). PCR conditions for both 1st and 2nd PCR cycles were 94℃ for 4 min, +94℃, 60 s/58℃, 60 s/72℃, 3 min ×35 cycles +72℃, 7 min +25℃. In addition, the fluorescence-modified cy5 at the 5' terminal of the 2nd primer was used as the sequence primer. Amino acid sequences were determined from the codons of obtained base sequences.

2.6. Ethical considerations

The present study conformed to the guidelines of epidemiological studies devised by the Ministry of Health, Labor and Welfare, Japan, to prevent leakage of personal information, and were conducted for the purposes of social benefit.

3. Results

3. 1. Backgrounds of patients at the start of lamivudine administration (Table 1)

Mean HBV-DNA was ranging from 5.8 to 6.9 LGE/mL, and mean ALT level, from 33 to 471 IU/L. Only the patient with liver cirrhosis was positive for HBeAg. In all cases, genotype was C. In 4 of the 5 patients, the YMDD motif was the wild-type "YMDD", but mutant-type "YVDD" was present in 1 patient who had been treated with low-dose intermittent administration of lamivudine prior to changing to the 100 mg/day treatment.

3. 2. Relationship between changes in YMDD motif and clinical course (Table 2)

Variant strains (YVDD) emerged in 2 of the 4 patients (50%; Patients 1 and 2) in whom the YMDD motif was the wild-type prior to the start of lamivudine administration. These mutants emerged at 45 months and 8 months, respectively, after the start of lamivudine administration. Within one month after lamivudine treatment was started, level of HBV-DNA in each patient fell below the limit of detec-

Table 1. Background data of the patients treated with lamivudine at the start of administration.

Patient	Sex	Age	Diagnosis	HBV-DNA (LGE/mL)	ALT (IU/L)	HBeAg	Anti-HBe	Genotype	YMDD Motif	Observation period (month)
1	Male	57	CH	6.7	471	(−)	(+)	C	YMDD	52
2	Male	49	LC§	5.8	33	(+)	(−)	C	YMDD	47
3*	Male	62	CH	6.1	81	(−)	(+)	C	YVDD	37
4	Male	65	CH	6.9	56	(−)	(+)	C	YMDD	46
5	Male	47	CH	5.8	62	(−)	(+)	C	YMDD	13

§ with jaundice and ascites

* treated with lamivudine 150 mg twice to 3 times a week for 32 months followed by conventional 100 mg daily administration

Table 2. Relationship between changes of YMDD motif during follow-up and HBV-DNA amount, ALT level and HBeAg/anti-HBe status at the start and end of observation.

Patient	YMDD Motif	HBV-DNA (LGE/mL)	ALT (IU/L)	HBeAg/anti-HBe
1	YMDD → YVDD*	6.7 → 3.7>	471 → normalized	(−)/(+) → (−)/(+)§
2	YMDD → YVDD** → YMDD	5.8 → 3.7>	33 → 87	(+)/(−) → (±)/(−)§§
3	YVDD → YVDD	6.1 → 6.1	81 → 162	(−)/(+) → (−)/(+)
4	YMDD	6.9 → 3.7>	56 → normalized	(−)/(+) → (−)/(+)
5	YMDD	5.8 → 3.7>	62 → normalized	(−)/(+) → (−)/(+)

* 45 months after the start of administration

** 8 months after the start of administration

§ with decrease of HBsAg titer

§§ with disappearance of jaundice and ascites

tion, 3.7 LGE/mL, and within 3 months ALT levels had also normalized.

Even though the mutant strain emerged in Patient 1, the amount of HBV-DNA dropped to the limit of detection level (3.7 LGE/mL) one month after the start of lamivudine administration and remained below that level. ALT also became normal 2 months after lamivudine administration and HBsAg titer was observed to decrease gradually. In Patient 2, who also developed a mutant strain, the amount of HBV-DNA dropped to the undetectable level 2 months after lamivudine administration and continued to be depressed below the limit of detection. ALT became almost stable and HBeAg titer was observed to decrease. In addition, jaundice and ascites disappeared in this patient with the improvement of serum albumin concentration and platelet count in peripheral blood leading to marked improvements in clinical symptoms.

In Patient 3, in whom the mutant strain (YVDD) had emerged prior to the start of administration of lamivudine at 100 mg/day, the amount of HBV-DNA dropped to the undetectable level immediately after lamivudine administration, however, it began to increase again after 6 months of treatment, and fluctuated in the range of 4.0−6.1 LGE/mL after the breakthrough. Moreover, at 18 months and 24 months after the start of lamivudine administration, ALT level elevated to 162 IU/L and 136 IU/L, respectively.

In Patients 4 and 5, in whom the wild-type YMDD motif persisted, the amount of HBV-DNA dropped to the undetectable level one month after treatment and remained below the limit of detection and ALT also continued to be normal with the fall of the amount of HBV-DNA.

3. 3. Relationship between changes in amino acid sequences in domain B and the YMDD motif (Table 3)

Each of the variants in the YMDD motif that emerged was "YVDD". In Patient 2, an LMA mutation emerged in domain B, and this predated the emergence of YVDD by 1 month. Thereafter, the LMA mutation persisted, but YVDD reverted to YMDD wild-type 2 months after emergence. In Patient 1, domain B showed an LLT variant at the initiation of lamivudine administration, but this reverted to LLA wild-type at 15 months after the start of lamivudine administration. YVDD subsequently emerged together with an LMA variant in domain B, and this pattern persisted thereafter. Patient 3 continued to show an LMA variant in domain B and YVDD during follow-up.

4. Discussion

The polymerase/reverse transcriptase region of HBV-DNA encodes for RNA-dependent DNA polymerase and is involved in viral

Table 3. Patterns of changes in amino acid sequences in domain B and YMDD motif in patients with and without emerging variant strains during follow-up.

Patient	Domain B−YMDD motif
1	LLT*-YMDD → LLA-YMDD → LMA**-YVDD
2	LLA-YMDD → LMA***-YMDD → LMA-YVDD → LMA-YMDD
3	LMA-YVDD
4	LLA-YMDD
5	LLA-YMDD

* threonine, ** 45 months after the start of administration, *** 1 month prior to YVDD emergence

proliferation due to reverse transcriptase activity. This region contains 5 conserved domains, A through E. Lamivudine-resistant viral strains display mutations in the tyrosine (Y), methionine (M), aspartate (D), aspartate (D) motif (YMDD motif) in the C domain (aa 548～558). Those variations comprise YIDD (M552I), in which the methionine is replaced by isoleucine, and YVDD (M552V), in which the methionine is mutated to valine. The YVDD variant often carries mutations not only in the YMDD motif, but also in the B domain, in which leucine is replaced by methionine (L528M). This B domain mutation reportedly results in even stronger resistance to lamivudine and is involved in aggravation of hepatitis.[7]

In both of our 2 patients in whom mutant strains emerged during treatment with lamivudine, HBV-DNA level continued to be depressed below the limit of detection and clinical symptoms improved despite emergence of a variant strain. One of these patients even reverted to the wild-type YMDD motif. Thus, even if an YMDD variant strain emerges, we should not immediately conclude that lamivudine has become ineffective and discontinue administration or start coadministration of other drugs. Rather, lamivudine treatment should be continued and course of the patient should be monitored.

In the 2 patients in whom a YMDD variant did not emerge, HBV-DNA level was continuously decreased below the limit of detection and ALT level was continuously normalized. These findings suggest that lamivudine therapy is generally useful for cases of chronic type B liver diseases. However, in the patient who displayed a mutant strain even before starting lamivudine therapy, HBV-DNA level was not continuously decreased, and ALT level repeatedly showed mild increases. If ALT level continues to show fluctuation in the future, consideration will be given to a multidrug regimen including interferon.[8]

Lok et al.[9] reported that the frequency of emergence of YMDD mutant strains was 23% in the first year and 65% after 5 years, and that the incidence of disease aggravation was significantly higher in patients in whom a mutant strain emerged. However, no great difference in the clinical course of patients has been identified in patients with emergence of a YMDD variant strain, regardless of whether lamivudine therapy was continued.[10] Consensus is thus lacking on this issue. The nucleoside analogs adefovir[11] and entecavir[12] have also been confirmed as effective against lamivudine-resistant viruses. In future, these agents are likely to become powerful treatment options for breakthrough hepatitis, either in combination with or without of lamivudine.

Further details are in order regarding those of our patients who showed marked improvement in clinical symptoms. Patients in whom YMDD variant strains emerged comprised 1 patient with anti-HBe positive chronic hepatitis and 1 patient with HBeAg positive decompensated liver cirrhosis. In addition, both patients who displayed no emergence of a YMDD variant were anti-HBe positive. Rizzetto et al.[13] reported that lamivudine therapy was effective in HBeAg negative patients even if a YMDD mutant emerged, and our experience suggests the same. Moreover, Ooga et al.[14] reported that lamivudine was useful in the treatment of some patients with cirrhosis of the liver if the disease had not progressed too far. However, one of our patients displayed decompensated liver cirrhosis showing ascites and jaundice, but lamivudine therapy still brought about marked improvement in clinical symptoms. When no other therapeutic option is available, lamivudine therapy is worth trying even for patients with liver cirrhosis that has progressed.

Changes in the combination of amino acid sequences in domain B and the YMDD motif were investigated in patients in whom YMDD

mutants emerged after the start of lamivudine administration. In Patient 1, domain B showed a rare LTT variant before lamivudine administration, and simultaneous emergence of LMA and YVDD was noted after starting lamivudine administration. In Patient 2, LMA persisted, but the emergence of YVDD was transient. For this type of domain B and YMDD motif, a discrepancy in the timing of emergence for the variant strain might represent some kind of regulatory factor in relation to the therapeutic effect of lamivudine. This may need to be investigated further in future studies. For Patient 3 who displayed YMDD mutant strains even before the start of lamivudine administration, prior intermittent low-dose lamivudine therapy might have been caused the emergence of mutant strains. In that light, daily administration of lamivudine appears essential for preventing emergence of YMDD mutant strains. Matsuda et al.[15] pointed out that almost no cases of YMDD mutant strains have been described for patients who have not been treated with lamivudine.

Treatment of patients with chronic type liver diseases by administration of lamivudine involves many important considerations. For example, if emergence of a YMDD mutant strain occurs, should lamivudine administration be continued? Or should a multidrug regimen including agents such as adefovir be implemented? And if no emergence of a YMDD mutant strain is seen and the clinical condition of the patient has stabilized, should lamivudine administration be discontinued? The most effective therapeutic regimen needs to be selected on the basis of such considerations. Kobayashi et al.[16] noted differences in the therapeutic efficacy of lamivudine as a function of genotype, and Matsumoto et al.[17] found that the therapeutic efficacy of lamivudine should be judged from a more comprehensive perspective of preventing carcinogenesis in chronic type B liver diseases. We look forward to further accumulation of knowledge in this regard.

References

1) Dienstag JL, Perrillo RP, et al.: A preliminary trial of lamivudine for chronic hepatitis B infection. N Engl J Med 333, 1657-1661, 1995.
2) Dienstag JL., Goldin RD., et al.: Histological outcome during long-term lamivudine therapy. Gastroenterology 125 1286-7, 2003.
3) Ling R, et al.: Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. Hepatology 24, 711-713, 1996.
4) Akuta N, Suzuki F, et al.: Virological and biochemical relapse according to YMDD motif mutant type during long-term lamivudine monotherapy. J Med Virol 504-510, 2003.
5) Kamisango, K., Kamogawa, C. et.al.: Quantitative detection of hepatitis B virus by transcription-mediated amplification and hybridization protection assay. J Clin Microbiol 37, 310-314, 1999.
6) Mizokami, M., Nakao, T. et al.: Hepatitis B virus assignment using restriction fragment length polymorphism patterns. FEBS Lett 450, 66-71, 1999.
7) Ono SK., Kato N., et al.: The polymerase L528M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. J Clin Invest 107, 449-55, 2001.
8) Suzuki F., Tsubota A., et al.: Interferon for treatment of breakthrough infection with hepatitis B virus mutants developing during long-term lamivudine therapy. J Gastroenterol 37, 988-90, 2002.
9) Lok AS., Lai CL., et al.: Long-term safety of lamivudine treatment in patients with chronic hepatitis B. Gastroenterology 126, 1932-3, 2004.

10) Liaw YF., Chien RN., et al.: No benefit to continue lamivudine therapy after emergence of YMDD mutations. Antivir Ther 9, 257-62, 2004.
11) Perrillo R. Schiff E, et al.: Adefovir dipivoxil for the treatment of Lamivudine-resistant hepatitis B mutants. Hepatology 32, 129-34, 2000.
12) Levine S., Hernandez D., et al.: Efficacies of entecavir against lamivudine-resistant hepatitis B virus replication and recombinant polymerases in vitro. Antimicrob Agents Chemother 46, 2525-32, 2002.
13) Rizzetto M.: Efficacy of lamivudine in HBeAg-negative chronic hepatitis B. J Med Virol 66, 435-51, 2002.
14) Ooga H., Suzuki F., et al.: Efficacy of lamivudine treatment in Japanese patients with hepatitis B virus-related cirrhosis. J Gastroenterol 39, 1078-84, 2004.
15) Matsuda M., Suzuki F., et al.: Low rate of YMDD motif mutations in polymerase gene of hepatitis B virus in chronically infected patients not treated with lamivudine. J Gastroenterol 39, 34-40, 2004.
16) Kobayashi M., Suzuki F., et al.: Response to long-term lamivudine treatment in patients infected with hepatitis B virus genotypes A, B, and C. J Med Virol 78, 1276-1283, 2006.
17) Matsumoto A., Tanaka E., et al.: Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B: A multicenter retrospective study of 2795 patients. Hepatol Res 32, 173-84, 2005.

和文要旨

Lamivudine 100 mg／日を経口投与し，1年以上の観察が可能であったB型慢性肝炎4例および非代償性肝硬変1例の計5例を対象とし，変異ウイルスの出現頻度および変異のパターンと臨床経過の関連について検討した．対象の内訳は全例男性，平均年齢は55.0±6.9歳（47～65歳），平均観察期間は39.0±15.5カ月である．治療前のHBV-DNA 量は5.8～6.9 LGE/mL，ALT 値は33～471 IU/L に分布し，HBe 抗原陽性は肝硬変の1例のみで，genotype は全例Cであった．Lamivudine 投与開始前の YMDD motif は5例中4例が “YMDD” の wild type であったが，少量間欠投与から継続して投与を行った1例は “YVDD” の mutant type であった．Lamivudine 投与開始前 YMDD motif が野生株であった4例中2例（50%）に，投与開始後それぞれ45カ月，8カ月に YVDD 変異株の出現をみた．Lamivudine 投与後は，全例1カ月以内に HBV-DNA 量は3.7 LGE/mL 未満の検出感度以下に低下し，3カ月以内までに ALT 値も正常化した．YVDD 変異株の出現をみた3例中2例は，変異株の出現にもかかわらず，HBV-DNA 量が検出感度以下を持続し ALT 値の正常化や病態の安定をみたが，他の1例はその後の経過中2回，HBV-DNA 量の再上昇を伴う ALT 値の増悪をみている．YMDD motif が野生型を持続した2例はいずれも，HBV-DNA 量は検出感度以下を持続し ALT も正常値を持続している．YVDD 変異株出現例の経過をみると，domain B の変異の消長と YMDD の変異株の消長との間に時間的位相のずれがみられる場合が有り，このことが Lamivudine の治療効果に影響を与えている可能性が示唆された．